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Regulation of mGluR1 expression in human melanocytes and melanoma cells

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1. INTRODUCTION

We reported earlier on a transgenic mouse line (TG-3) that develops spontaneous melanoma with 100% penetrance. The resultant tumor phenotype in these transgenics is a classical example of insertional mutagenesis in which a single insertion of a transgene [1, 2] led to a deletion of approximately 70kb of the host sequences on mouse chromosome 10. This mouse chromosomal region is syntenic to human chromosome 6q on which a large number of human, non-familial malignant melanomas display rearrangements [3, 4]. The deleted 70kb host region was identified as part of intron 3 of the gene encoding metabotropic glutamate receptor 1 (gene: mouse, *Grm1*, human, *GRM1*, protein: mGluR1). In order to definitively demonstrate that *Grm1* had a direct etiological role in melanoma development in the TG-3 transgenic line, we generated a new transgenic line with wild type mouse *Grm1* cDNA under the regulation of a melanocyte-specific promoter, Dopachrome Tautomerase (*Dct*). The resulting pigmented tumors in the founder and subsequent progeny of this new transgenic line (line E) were very similar to the original TG-3 transgenics [5]. These findings unequivocally demonstrated that the introduction of *Grm1* cDNA alone into mouse melanocytes was sufficient to induce melanocytic neoplasia *in vivo*. Ohtani and co-workers confirmed our findings recently by engineering an inducible *Grm1* transgenic mouse model in which induction of *Grm1* expression in adult mice resulted in melanoma development in 100% of the mice [6]. To-date we have tested more than 175 human melanoma biopsies and 32 human melanoma cell lines and detected mGluR1 at both RNA and protein levels in about 60% and 80% respectively of these samples. The functionality of mGluR1 in human melanoma cells has also been demonstrated by specific cellular responses to agonist and antagonists of mGluR1 [5, 7]. Recently, other glutamate receptors have also been shown to have a role in melanoma pathogenesis including *GRM3*, *GRM5* and *GRIN2A* [8–10]. These exciting results are strong indications that glutamate signaling plays a more significant role

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than previously known in melanoma biology and elucidating the mechanisms regulating the expression of these genes is crucial in understanding this deadly disease.

mGluR1, a member of the metabotropic glutamate receptors, is a G-Protein-Coupled Receptor (GPCR) [11, 12]. GPCRs comprise the largest known family of cell surface receptors and mediate cellular responses to a diverse array of signaling molecules including hormones, neurotransmitters, and chemokines. Ectopic expression or constitutive activation of a variety of GPCRs has been implicated in neoplastic transformation such as the FSH receptor in ovarian cancer [13], CCK₂ receptor in colorectal cancer [14], and the neuromedin B receptor in small-cell lung cancer [14, 15]. Activating mutations are infrequent in GPCRs however; these receptors contribute to neoplastic transformation when persistently stimulated by their respective ligands that are released from surrounding tissues. The released ligands in turn bind to their respective receptors and establish autocrine and paracrine feedback loops that stimulate tumor growth [14, 16, 17]. Our group demonstrated the presence of enhanced levels of extra-cellular glutamate, the natural ligand of mGluR1, in several human melanoma cell lines positive for mGluR1 expression [7]. It is therefore not surprising that ectopic expression of mGluR1 in melanocytes can lead to oncogenesis.

The mechanisms by which transcription of *GRM1/Grm1* is activated in melanocytes are unknown. Earlier studies by others identified a Neuron-Restrictive Silencing Factor/Repressor Element Silencing Transcription Factor (NRSF or REST) as a master regulator of expression of neuronal specific genes in non-neuronal cells. NRSF is a Kruppel-type zinc finger transcription factor that interacts with a 23bp cis-acting Neuron-Restrictive Silencer Element/Repressor Element 1 (NRSE or RE1) to suppress neuron-specific gene expression in non-neuronal cells [18, 19]. Several recent reports have revealed that NRSF may have other functions, including roles in both tumor suppression and progression in addition to its regulation of neuronal gene expression in non-neuronal cells [20–23]. Upregulation of NRSF expression has been demonstrated in medulloblastoma and NRSF isoforms with modified repression domains have been reported in small-cell lung cancer, colon cancer and neuroblastoma [20–23]. These results suggest that regulation of expression of neuronal-specific genes by NRSF is likely to be complex and through multiple pathways. In both non-neuronal and neuronal cells, NRSF silences the expression of its target genes through its two independently acting repressor domains [18, 19]. The N-terminal repressor domain of NRSF recruits co-repressors, such as mSin3A and histone deacetylases (HDACs) into the vicinity of the promoter. Histone deacetylation leads to a more compact chromatin that prevents accessibility of transcription factors. The C-terminal repressor domain (CTRD) of NRSF has been shown to interact with at least one factor, the transcriptional co-repressor (CoREST) that may serve as a platform protein for the recruitment of molecular machinery that imposes silencing across a chromosomal interval [24, 25]. CoREST is able to interact with HDAC1/2, indicating that the CTRD also contributes to histone deacetylation [26].

Ferraguti and colleagues have identified a consensus NRSE site within 5kb upstream of the *Grm1* initiating codon and have shown that the lack of mGluR1 expression in BHK and NIH3T3 cells is mediated by the binding of NRSF to NRSE at the 5' region of *Grm1* [27]. Based on these studies, we hypothesized that aberrant expression of mGluR1 in melanoma cells derives from failure of NRSF binding to NRSE and/or recruitment of necessary co-factors. Here, we show that in human melanoma cells, NRSE within the *GRM1* promoter region is wild type and that NRSF is only partially functional. In addition, we also present evidence that in addition to lack of binding of NRSF to NRSE, Sp1 binding to the GC box bordering NRSE within the human *GRM1* promoter is also involved in modulating mGluR1 expression in melanocytes. Finally methylation at the *GRM1* promoter region may also play a role in the regulation of mGluR1 expression in melanocytes.

2. MATERIALS and METHODS

2.1 Cell culture, transient transfection, mithramycin A or 5-azacytidine treatment and luciferase assays

Primary human epidermal melanocytes (HEM) were purchased from Cascade Biologics (Portland, OR), FOM 139-1 were from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA), immortalized human melanocytes, hTERT/CDK4^{R24C}/p53^{DD} were from Dr. David Fisher (Harvard Medical School, Boston, MA) [28]. All melanocytes were maintained in Medium 254 with human melanocyte growth supplement (Invitrogen, Carlsbad, CA). Human melanoma cell lines WM239A, WM8, WM1382, WM3211, C8161, C81-61, SKMEL 187, UACC903, UACC930, MV3 and M19 lines were from Drs. Meenhard Herlyn (Wistar Institute, Philadelphia, PA), Mary Hendrix (Northwestern University, Chicago, IL), Dr. Alan Houghton (Memorial Sloan Kettering, New York, NY), Dr. Jeffrey Trent (TGen, Phoenix, AZ) and Dr. Juergen Becker (University Hospital for Dermatology and Venerology, Graz, Austria), all melanoma cells were maintained in RPMI 1640 plus 10% fetal bovine serum (FBS).

For luciferase assays, luciferase constructs were transfected using DOTAP (Roche, Basel, Switzerland) according to manufacturer's instructions. At 48 hours post-transfection, cell lysates were collected and firefly and Renilla luciferase activity levels quantified using the Dual-Glo luciferase assay system (Promega, Madison, WI) according to the manufacturer's specifications by a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA).

Melanoma cells were treated with 100nM or 500nM of mithramycin A (Tocris, Ellisville, MO) for 24 hours and protein lysates collected and analyzed by Western blotting for mGluR1 expression (Novus Biologicals, Littleton CO). Sp1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad CA). Protein lysates were collected 72 hours post transfection and analyzed by Western blotting with anti-Sp1 antibody (Santa Cruz, CA) and mGluR1 antibody (Novus Biologicals, Littleton CO). To demonstrate equal loading, antibody to α -Tubulin (Sigma, St. Louis, MO) was used. For 5-azacytidine treatments, the cells were treated at 70% confluency with 10 μ M 5-azacytidine (Sigma, St. Louis, MO) for specified time periods followed by protein extraction. MAGE antibody was purchased from Millipore (Billerica, MA). PCR band purification was performed with PCR Purification kit (Agilent Technology, Santa Clara, CA). DNA Sequencing was performed at the DNA Core Facility, Rutgers University.

2.2 Plasmid constructs and mutagenesis

Dr. Ferraguti provided us with information on 5kb of DNA sequences 5' of the initiation codon of the human *GRM1* gene. Designating the transcription initiation site in exon Ia as +1, the region delimited by +585/NRSE/+1183 or +585/NRSE/Sp1/+1183 was amplified from the genomic DNA of human C8161 cells by PCR using the oligonucleotide primers: 5'-GTGTGTGTGTGTGTGTATGGGTAA -3' and 5'-TTGGGTTCTCTTGAAAGGCCTAA -3'. This fragment was then subcloned into the pGL3-basic vector. Mutations in the NRSE and/or Sp1 sequences of the *GRM1* promoter were generated by PCR site-directed mutagenesis using high fidelity *Pfu* DNA polymerase according to the manufacturer's protocol (QuickChange™; Stratagene, La Jolla, CA). *In vitro* mutagenesis was carried out on NRSE and/or Sp1 site in *GRM1* promoter linked to luciferase reporter gene (hGRM1NE and/or hGRM1NESP) using primers as follows:

hGRM1NE(=hGRM1NESP):

5' GACCGAGCACTGTCCAGGGTCCTGAAGGCGGCAGGGCTCAGG - 3'. 5'-
CCTGAGCCCTGCCGCCTTCAGGACCCTGGACAGTGCTCGGTC'-3.

hGRM1NEm:5'-
 GACCGAGCACTATGTATTGGTCCTGAAGGCGGCAGGGCTCAGG - 3'.
 5'- CCTGAGCCCTGCCGCCTTCAGGACCATACATAGTGCTCGGTC - 3'.
 hGRM1NESpm:5'-
 GACCGAGCACTGTCCAGGGTCCTGAAGATGATAGGGCTCAGG - 3'.
 5'- CCTGAGCCCTATCATCTTCAGGACCAATACATAGTGCTCGGTC - 3'.
 hGRM1NEmSPm:5'-
 GACCGAGCACTATGTATTGGTCCTGAAGATGATAGGGCTCAGG-3'. 5'-
 CCTGAGCCCTATCATCTTCAGGACCAATACATAGTGCTCGGTC-3'.

Underlining denotes the mutated nucleotides for NRSE and Sp1 binding sites. All constructs were verified by DNA sequencing.

2.3 RNA preparation, RT - PCR

RNA isolation was performed using TRI reagent (Molecular Research Center, Cincinnati, OH). RNA (2–5µg) was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using random hexamers as primers. PCRs were performed with recombinant *Taq* DNA polymerase and primers. Primer sets used for *GRM1* were sense, 5'-TCCACACGGAAGGGAATTAT-3'; antisense, 5'-GCAAGAGTCGGTCAAAGCTC-3'; while the GAPDH was amplified with the primer set; sense, 5'-GAGTCAACGATTTGGTCGT-3'; antisense 5'-TGTGGTCATGAGTCCTTCCA-3'.

2.4 Protein analysis

Human tissues were obtained from Tissue Retrieval Facility Core at the Cancer Institute of New Jersey. Protein extraction and Western immunoblots were performed as described earlier [5, 7]. Digitized images of exposed X-ray films were analyzed with Optiquant Software (Packard Instruments, Meriden, CT) with the ratio determined from intensities of each protein to respective loading control.

2.5 Nuclear protein extract preparation and Co-immunoprecipitation

Nuclear protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic extraction kit (Pierce Biotech, Rockford, IL). For co-immunoprecipitation experiments, 100µg of nuclear extracts were incubated with 2.0µg of the appropriate antibodies (Sp1, Sp2, Sp3 from Santa Cruz Biotech.) for 24h at 4°C in 500µl of immunoprecipitation buffer (20 mM Hepes-KOH, pH 7.9, 25% Glycerol, 420mM NaCl, 1.5 mM MgCl₂, 0.2mM EDTA, 0.5mM dithiothreitol, and 0.2mM phenylmethylsulfonyl fluoride) [29]. Protein A-agarose beads (25µl) were used to precipitate the associated protein. Samples were then separated by SDS-PAGE and detected by Western blot analysis using the anti-NRSF antibody (Upstate Biotech, Billerica, MA).

2.6 Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed using the Upstate ChIP assay kit. Antibodies were purchased from the following vendors: NRSF, CoREST and HDAC1 (Upstate Biotech., Billerica, MA), mSin3A and Sp1 (Santa Cruz Biotech., Santa Cruz, CA). The PCR NRSE primers were: NRSE forward, 5'-GTGTGTGTGTGTGTATGGGTAAAG-3'; NRSE reverse, 5'-GTGGGTGGACGGGTTAGG-3'.

2.7 Promoter methylation PCR

Promoter methylation PCR assays were done using the Promoter methylation PCR kit (Panomics, Redwood City, CA). Restriction enzyme MseI was purchased from NE Biolabs (Ipswich, MA). Genomic DNA was isolated as described [1]. PCR primer sets; set 1 (−4846/−5267) sense, 5'-CTGATGACAAGGTTGTGATTTTTC-3'; antisense, 5'-AGAAGGGACACCTCCAAAAAGAT-3'. Set 2 (−3257/−3874) sense, 5'-TAGATAAGTTTTTGGGTGATTGTT-3'; antisense, 5'-ATTTTTATTATTTTGTATTGTTT-3'.

3. RESULTS

3.1 NRSF expression in human melanoma cells

We have identified that about 80% of human melanoma cell lines and 60% of biopsy samples tested were positive for mGluR1 expression [5, 7]. An example of the mGluR1 expression in several human melanoma biopsies, dysplastic nevi and nevi biopsies and primary human epidermal melanocytes (HEM) is shown (Figure 1A). Expression of mGluR1 is detected in most of the human melanoma biopsies tested and at a lower level in dysplastic nevi and even lower in nevi, but not detectable in HEM (Figure 1A). We hypothesized that aberrant expression of mGluR1 in human melanomas may be a consequence of mutation(s) in NRSF and/or NRSE resulting in the absence of binding of NRSF to NRSE. Levels of NRSF were then examined in human melanoma cell lines and biopsies that are either positive or negative for mGluR1 expression. We detected varying levels of NRSF expression by Western immunoblots (Figure 1B) and real-time quantitative RT/PCR (data not shown) with no apparent correlation to mGluR1 expression. Next, we analyzed possible mutation(s) in NRSE using the same set of samples from the NRSF experiments as templates to amplify the NRSE region by PCR followed by DNA sequencing. A consensus NRSE site 5' upstream of the *GRM1* initiation codon within the intronic region between exon Ia and II of human *GRM1* promoter was identified previously by Ferraguti and co-workers [27]. All samples examined displayed wild type NRSE sequences (data not shown).

These results were not anticipated but not completely unexpected considering several groups have reported similar discrepancies in other cancers [20, 21, 30]. In medulloblastoma and small-cell lung cancer, higher levels of NRSF were detected in tumors as compared to the respective normal counterpart tissues [20, 21, 30]. These groups also identified novel splice variants of NRSF (16–62 bp insertions) in small-cell lung cancer and neuroblastoma. One or more of these variants antagonize repression of neuronal genes mediated by NRSF [20, 22, 31]. Of these variants, REST4 has been identified in a subset of small-cell lung cancer where it functions as a dominant negative mutant to NRSF thus preventing the binding of NRSF to NRSE resulting in aberrant expression of neuronal specific genes [20, 32]. We next examined if any of the novel splice variants of NRSF with small insertions (16–62 bp) and REST4 were present in human melanoma cell lines and biopsies by RT-PCR followed by DNA sequencing. We did not identify any of the novel splice variants previously described (data not shown), concurrently, REST4 expression was only identified in the positive control IMR32 neuroblastoma cell line (Figure 1C).

3.2 NRSF is only partially functional in C8161 and UACC903 human melanoma cells

To determine the functionality of NRSF binding to NRSE within the *GRM1* promoter, we generated recombinant luciferase reporter constructs containing either wild type (hGRM1NE) or mutated NRSE motifs (hGRM1NE_m). hGRM1NE or hGRM1NE_m were transiently transfected into the mGluR1-positive human melanoma cell lines C8161 and UACC903, mGluR1-negative human embryonic kidney cells HEK293 and primary human

epidermal melanocytes (HEM). Introduction of mutated NRSE (hGRM1NEm) into HEK293 or HEM resulted in increased promoter activity as indicated by the increase in luciferase activity compared to that of wild type NRSE (hGRM1NE) (Figure 2A). In contrast, promoter activity was only slightly increased with mutated NRSE (hGRM1NEm) compared to wild type NRSE in mGluR1-expressing C8161 or UACC903 (Figure 2A). These results imply that mutation(s) within the NRSE motifs relieve the binding of NRSF within the *GRM1* promoter region in HEK293 and HEM cells while in C8161 and UACC903 cells, mutated NRSE only slightly alleviated NRSF binding within the *GRM1* promoter region suggesting that in C8161 or UACC903 melanoma cells, other factor(s) may be necessary in addition to NRSF binding to NRSE in NRSF-mediated regulation of mGluR1 expression.

Earlier studies have shown that additional factors including co-repressors, such as mSin3A [33, 34] and CoREST [26, 35] are required to interact with NRSF for it to elicit its repressive activity. This complex, in turn, recruits multiple co-factors and transcriptional regulators including histone deacetylases (HDACs) [18, 19, 24]. Expression of these co-repressors was assessed by Western immunoblots and expression of both CoREST and mSin3A was detected in all human melanoma cell lines and biopsies tested (Figure 2B). We also performed RT/PCR to assess the expression of three other known NRSF regulated genes; *SCG-10* [36, 37], *Syn1* [38–40] and *GluR2* [33, 41, 42] in neuronal and non-neuronal cell lines. The three mRNAs were only expressed in the neuronal cell line (IMR-32) and not in the non-neuronal cell lines (Figure 2C). Taken together, these results suggest that all the melanoma cells and biopsy samples tested showed expression of co-repressors that are required for NRSF function (Figure 2B) and that not all NRSF regulated neuronal genes are deregulated in the non-neuronal cells examined (Figure 2C). It thus appears that the expression of mGluR1 in some human melanoma cells is likely to be a specific deregulation event in these cells.

Next, we performed chromatin immunoprecipitation assays with normal human melanocytes, HEM, and two mGluR1-positive human melanoma cell lines, UACC903 and C8161 to examine protein-DNA interactions within the *GRM1* promoter region. After the cross-linking of proteins and DNA with formaldehyde, the cell lysates were subjected to immunoprecipitation with antibodies against NRSF and HDAC1. The precipitated DNA fragments were then amplified with primers spanning the 226bp NRSE site on the *GRM1* promoter. ChIP-PCR products from HEM, UACC903 and C8161 cell lysates were detected with the anti-NRSF antibody as well as anti-HDAC1 antibody, but not detected with normal rabbit IgG control (Figure 2D). These results suggest that in HEM, UACC903 and C8161 cells, the endogenous NRSF and its co-repressor HDAC1 specifically bind to the NRSE region on the *GRM1* promoter. Taken together, these findings propose that additional repression mechanisms mediated by NRSF may be required to repress mGluR1 expression in human melanoma cells.

3.3 Sp1 interacts with NRSF and binds to the GC box next to NRSE in C8161 human melanoma cells

Computational searches using the BLAST database were performed to identify putative regulatory elements that may be involved in the regulation of mGluR1 expression in melanocytes. We identified a GC box sequence adjacent to NRSE on the *GRM1* promoter (Figure 3A). GC box sequences are recognized as consensus nucleotide-binding sites for the Sp1 transcription factor in most NRSF target genes [29]. Studies have confirmed the functional implication of Sp1 in expression of N-methyl-D-aspartate receptors, dynamin I, synaptophysin, and ionotropic kainate-5 genes [43–47]. The possibility that suppression of mGluR1 expression in melanocytes requires the inhibition of Sp1-dependent regulation of mGluR1 expression was evaluated next.

We assessed the physical interaction of endogenous Sp1 and NRSF by co-immunoprecipitation and Chromatin immunoprecipitation (ChIP) assays in C8161 human melanoma cells and normal human primary melanocytes (HEM). Nuclear extracts from C8161 and HEM were immunoprecipitated (IP) with antibodies against Sp1, Sp2, Sp3, and NRSF. Normal rabbit IgG was used as a negative control. These IP samples were then probed with NRSF antibody. The endogenous Sp1 factor was found co-immunoprecipitated with NRSF only in C8161, not in HEM (Figure 3B). These results imply that endogenous Sp1 interacts with endogenous NRSF and may be involved in regulating mGluR1 expression in C8161 melanoma cells whereas NRSF does not appear to interact with Sp1 in normal melanocytes, HEM.

To validate Sp1 binding to the *GRM1* promoter in human melanoma cells, we took advantage of the antibiotic, mithramycin A. Mithramycin A is known to bind to GC-rich DNA sequences, thus preventing Sp1 from binding to the same sites and as such, it is frequently used to analyze the sequence dependency of DNA binding factors [48, 49]. Four mGluR1 expressing melanoma cell lines; C8161, UACC903, MV3 and M19 were treated with 100nM or 500nM of mithramycin A for 24 hours and protein lysates analyzed for mGluR1 expression. Elevated levels of mGluR1 protein were detected in mithramycin A treated melanoma cell lines with only M19 showing a dose dependent increase in mGluR1 expression (Figure 3C). To ascertain that these modulations in mGluR1 expression levels observed were the consequences of inhibition of Sp1 binding the GC rich DNA sequence, we utilized Sp1 specific silencing RNA to knock down Sp1 in C8161 and M19 melanoma cells. Levels of Sp1 were reduced within 72 hours in the presence of siSp1 RNA (Figure 3D, left panel) and enhanced mGluR1 expression was also detected (Figure 3D, right panel). Taken together, these results suggest that Sp1 acts as a negative regulator of mGluR1 expression in human melanoma cells.

3.4 Inhibition of Sp1 binding to the GC box recovered partial NRSF function in human melanoma cells

To further assess if the binding of Sp1 to the GC box next to the NRSE site on the *GRM1* promoter plays a pivotal role in the regulation of mGluR1 expression in C8161 or UACC903 human melanoma cells, we generated the following NRSE-Sp1 linked luciferase reporter constructs: wild type at both NRSE and Sp1 binding sites (hGRM1NESP); mutated NRSE binding site (hGRM1NEmSP); mutated Sp1 binding site (hGRM1NESPm); and mutated at both NRSE and Sp1 binding sites (hGRM1NEmSPm) (Table 1). Each of the constructs was transiently transfected into C8161, UACC903, HEM and HEK293 followed by assessment using Dual-luciferase reporter assays. We found that the binding of NRSF to NRSE was inhibited when the NRSE binding site was mutated (hGRM1NEmSP) resulting in increased luciferase activity in all four cell lines compared to wild type NRSE and Sp1 (Table 1), but much more so in HEM and HEK293 cells as observed earlier (Figure 2A). In HEM and HEK293 cells, mutated Sp1 binding site (hGRM1NESPm), alone resulted in a slight reduction of luciferase activity (Table 1). In contrast, both C8161 and UACC903 showed a drastic decrease in *GRM1* promoter activity compared to wild type NRSE and Sp1 binding sites (hGRM1NESP) (Table 1). In HEM and HEK293 cells, mutations at both NRSE and Sp1 binding sites (hGRM1NEmSPm) yielded an increase of *GRM1* promoter activity, very similar results as when only NRSE binding site is mutated (hGRM1NEmSP), however, the enhanced *GRM1* promoter activity was more in HEK293 than HEM (Table 1). On the contrary, for C8161 and UACC903 cells, mutations at both NRSE and Sp1 binding sites (hGRM1NEmSPm) resulted in a moderate alteration in *GRM1* promoter activity (Table 1). Taken together, these results suggest that for UACC903 and C8161 melanoma cells, when both NRSF and Sp1 binding sites are mutated, binding of NRSF to NRSE is inhibited and contributed to a partial increase in *GRM1* promoter activity as evident by more

luciferase activity. Mutation at the Sp1 binding site adjacent to NRSE on *GRM1* promoter resulted in the inability of Sp1 to bind to the mutated GC box and led to a decrease in *GRM1* promoter activity. The net outcome due to double mutations at NRSF and Sp1 binding sites is intermediate de-repression of *GRM1* promoter activity (Table 1).

3.5 Methylation at the 5' region of *GRM1*

To investigate whether methylation of the *GRM1* promoter also has a role in the regulation of mGluR1 expression in melanoma cells, we utilized two different web-based programs, MethPrimer and MethDB and identified two putative CpG islands within 5kb of *GRM1* sequences upstream of the first ATG. Two different mGluR1-negative normal human melanocytic lines (hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1) were treated with a DNA demethylation reagent, 5-azacytidine for up to 9 days and cell extracts were collected on days 0, 3, 5, 7 and 9 and subjected to Western immunoblots for mGluR1 expression (Figures 4A and 4B). A known methylation-regulated gene in melanocytes and melanoma is the tumor antigen, MAGE A4. The demethylation of the promoter region of MAGE in melanocytes can be induced by 5-azacytidine and permits MAGE A4 expression [50]; thus MAGE A4 expression was used as a positive control for demethylation in Westerns (Figure 4A). We detected mGluR1 expression in both hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 cells starting at Day 3 after 5-azacytidine treatments. The positive control, MAGE A4 expression, was also detected from Day 3 onward. These results suggest that methylation at *GRM1* promoter could also regulate mGluR1 expression in melanocytes. To further verify these observations, we used a Methylation Promoter PCR kit (Panomics) that utilizes the ability of a protein, MeCP2 (methylation binding protein), to bind to methylated DNA regions only. The DNA-protein complex can then be separated from the unbound regions through spin columns. We isolated the DNA/MeCP2 complexes from both hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 cells and selectively amplified by PCR with specific primer sequences within the *GRM1* promoter region flanking the putative methylated CpG islands. The PCR products were analyzed by gel electrophoresis and the presence of a diagnostic band was indicative of methylation at the promoter region of *GRM1*. As shown in Figure 4C, visible PCR products of the expected sizes were detected in all four samples before treatment with MeCP2 in the Methylation Promoter PCR kit (lanes 2, 5, 8 and 11). Post-treatment with MeCP2 showed that only the primer set flanking *GRM1* promoter region bases -4846/-5267 exhibited an expected band size at 421bp (lanes 3 and 6) in both hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 cells. The lack of PCR bands (617 bp) in MeCP2 treated, MseI digested genomic DNA using the primer set flanking the *GRM1* promoter region (bases -3257/-3874) strongly suggests that this putative CpG island was likely not methylated. All PCR bands were isolated, purified and sequences confirmed by DNA sequencing.

4.0 DISCUSSION

Normal cellular function and developmental processes require temporal and spatial regulation of gene expression by the activity of transcription factors that bind to the regulatory elements of each gene. Alteration(s) and deletion(s) resulting in loss of activity of transcription factors have been linked to various human disorders including cancer. Studies have demonstrated the involvement of NRSF in several human cancers [51–53]. We have previously reported that ectopic expression of mGluR1 in melanocytes is sufficient to induce spontaneous melanoma development *in vivo* [5]. In addition, we also demonstrated aberrant mGluR1 expression in a subset of human melanoma cell lines and biopsies, suggesting that mGluR1 may be involved in some human melanomas [5, 7]. A critical question remains: how can the transcription of a neuronal gene, *GRM1*, become deregulated resulting in ectopic expression of mGluR1 in melanocytes resulting in a transformed phenotype?

Ferraguti et al., reported on the regulation of mGluR1 expression in neuronal and non-neuronal cells by NRSF/NRSE [27]. We were interested to know if such regulation occurs in melanocytes. First, we showed that in all melanoma cell lines and biopsies examined, NRSF is expressed and NRSE sequences are wild type. Differential expression of novel splice variants of NRSF in human melanoma cells was not detected regardless of mGluR1 expression. Using luciferase-linked reporter assays, we showed that mutated NRSE could relieve transcription-repressive activity more in HEM and HEK293 cells than UACC903 and C8161 melanoma cells, despite the interactions between endogenous NRSF and its co-repressor HDAC1 with NRSE (Figure 2). From these results, we hypothesized that additional player(s) along with NRSF may be required to regulate the expression of mGluR1 in human melanoma cells. Sequence analysis identified a conserved GC box adjacent to NRSE in the human *GRM1* promoter. Sp1 is known to be a transcriptional activator in the expression of NRSF target genes by binding to the GC boxes [29]. However, Sp1 has also been reported to be a negative regulator of other NRSF regulated genes [54]. Co-immunoprecipitation experiments showed that Sp1 interacts with NRSF in C8161, but not in normal human melanocytes, HEM (Figure 3B). The consequences of inactivation of Sp1 or NRSE and *GRM1* promoter activity were assessed with wild type or mutated NRSE/Sp1 linked luciferase reporter constructs. We showed that mGluR1 expression in HEM and HEK cells is predominantly regulated via binding of NRSF to NRSE as shown for BHK and NIH3T3 cells [27]. The failure of Sp1 binding to the GC box DNA sequences as assessed by either mithramycin A inhibition or silencing RNA to Sp1 led to enhanced mGluR1 expression (Figures 3C and 3D), in contrary, specific mutation at the Sp1 binding site within the *GRM1* 5' region led to a decrease in *GRM1* promoter activity (Table 1). It is well known that Sp1 is an essential factor for efficient initiation of transcription of genes by facilitating binding of Transcription Factor IID (TFIID) to the promoter, which begins the assembly of the pre-initiation transcriptional complex for RNA polymerase II-mediated transcription in the absence of a TATA box [55, 56]. The TATA box is bound by a protein complex, which contains TATA binding protein and associated factors. Inactivation of Sp1 results in suppression of TATA binding protein activities and early silencing of the transcriptional machinery [57–59]. Recent data have also shown that the Sp family of transcription factors can also serve as negative regulators of gene expression. Sp3 has previously been shown to act as repressor through its ability to recruit HDAC1 or 2 associated with the mSin3 [60, 61]. Our findings indicate that a general inhibitor of Sp1 such as mithramycin A or siSp1 that will suppress Sp1 activity in genes that have at least one GC box within the regulatory region resulting in an increase in mGluR1 expression, while precise mutations at the GC box within the *GRM1* promoter region only interferes with Sp1 function at that particular site, leading to a decrease in *GRM1* promoter linked reporter activity. To reconcile these contradictory observations, we hypothesize that there is at least one other factor that is involved in the regulation of mGluR1 expression in non-neuronal cells (such as melanocytes). This factor is negatively regulated by Sp1. When Sp1 activity is suppressed by mithramycin A or siSp1, this factor is not inhibited and thus mGluR1 expression occurs, however, when the Sp1 site is exclusively mutated on the *GRM1* 5' promoter region, a reduction in *GRM1* promoter activity is detected, likely from inhibition of this factor. The identity of this putative factor is currently unknown.

We also investigated the possibility of promoter methylation involvement in the regulation of mGluR1 expression by treating either primary or immortalized human melanocytes that lack mGluR1 expression [5] with a well-known DNA demethylating agent, 5-azacytidine. Our results showed that only one out of the two putative CpG islands in the promoter region is likely involved in the regulation of mGluR1 expression in melanocytes as indicated by the appearance of amplified PCR fragment after complex formation with the methylation binding protein, MeCP2. We demonstrated that primary human melanocytes (FOM 139-1) and an immortalized human melanocytic line (hTERT/CDK4^{R24C}/p53^{DD}) that have not

been extensively expanded in culture yielded identical results. To be absolutely certain that demethylation at this site is a critical step in human melanomagenesis one will need to systematically analyze possible methylation at *GRM1* promoter in a large number of melanoma specimens from early stage to late ones. Such an approach may be impractical to perform with melanoma cell lines that have been cultured by many investigators, as results obtained could be “tissue culture artifacts” and lead to un-interpretable outcomes.

In light of the results by other groups and our current studies, we propose that in transformed melanocytes (melanoma), binding of NRSF to NRSE in the *GRM1* promoter region is not sufficient to overcome the already suppressed Sp1 that has established the formation of the initiation transcriptional complex leading to the repressed expression of the NRSF target gene, *GRM1*. The factor(s) that may be involved in the inhibition of Sp1 in melanoma cells remains to be identified and possible involvement of promoter methylation also should be considered. These results highlight the importance of understanding the complex molecular regulation of tissue-specific genes especially in cancer where deregulation of these genes often leads to aberrant patterns of transcription.

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Highlights

Possible mechanisms in the regulation of mGluR1 expression in melanocytes include:

- Binding of NRSF to NRSE at 5' region of *GRM1*.
- Negative regulation by Sp1
- Demethylation at the *GRM1* promoter.

Figure 1.

A: Aberrant mGluR1 expression is detected in human melanoma biopsies and dysplastic nevi. HEM is included as normal melanocyte control. A melanocyte-specific marker, tyrosinase related protein 1 (TYRP1), is used as loading control. In HEM, the TYRP1 is not fully modified thus it migrates faster. The arbitrary units of the intensity of mGluR1 to TYRP1 were determined with Optiquant Software and are shown below each lane. **B:** NRSF expression in several melanoma cell lines and a subset of biopsies from panel A. IMR-32, a neuroblastoma cell line was included as a control. Varying levels of NRSF were detected among melanoma cell lines and biopsies; there was no correlation with aberrant mGluR1 expression. The arbitrary units of the intensity of NRSF to α-Tubulin were determined with Optiquant Software and are shown below each lane. **C:** Examination of REST and REST4 expression by RT/PCR on RNA isolated from human melanoma cell lines with primers that discriminate between full-length REST and its variant, REST4. IMR-32 is a neuroblastoma cell line and is used as a positive control for REST4. HEK293, a human embryonic kidney cell line, is used as a positive control for the full-length REST. mGluR1 expressing human melanoma cell lines are C8161, Skmel1187, WM239A and WM3211. mGluR1 negative melanoma cell lines are C81-61, UACC930, WM8, and WM1382. NHM and HEM are

normal human melanocytes. RT-PCR showed wild type REST (NRSF) and REST4 at 311 and 361bp, respectively. GAPDH was used as a control for equal amounts of template in RT/PCR. The numbers represent arbitrary units of intensity of Rest or Rest 4 to GAPDH.

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Figure 2.

A: NRSF represses *GRM1* gene transcription through an NRSE dependent mechanism. Wild type (hGRM1NE) and mutated NRSE (hGRM1NEm) linked luciferase constructs were transfected into C8161, UACC903, HEK293 and HEM. Promoter activity of each construct is expressed as luciferase activity relative to the activity of each corresponding luciferase reporter with pGL3 basic vector, which is assigned an activity value of 1. The data shown is the average of three independent experiments $\pm$ S.D. **B:** Examination of Co-REST and mSin3A expression by Western immunoblots on protein lysates isolated from mGluR1-negative HEK293 and normal human primary melanocytes (HEM), mGluR1 expressing human melanoma cell line C8161, and human biopsies: nevi (mGluR1 negative) and eight other mGluR1 expressing biopsies. α -Tubulin was used as a loading control. The numbers represent arbitrary units of intensity of coREST or mSin3A to α -Tubulin. **C:** Examination of three other known NRSF regulated genes by RT-PCR. Only IMR-32, a medulloblastoma cell line showed expression of all three, none was detected in the other four non-neuronal cell lines. **D:** NRSF binds to the NRSE region on the *GRM1* promoter and is detected by Chromatin Immunoprecipitation (ChIP) in C8161, UACC903 and HEM. Input is an aliquot of the DNA-protein complexes before immunoprecipitation. The remaining DNA-protein complexes are subjected to immunoprecipitation with antibodies against NRSF, HDAC1 and control rabbit IgG. Immunoprecipitated DNA was amplified with specific primers across the NRSE region of the *GRM1* promoter

Figure 3.

A: Putative Sp1 transcription factor binding GC box is detected adjacent to the NRSE site in the human *GRM1* promoter. **B:** Co-immunoprecipitation of the endogenous NRSF and Sp1 in C8161 and HEM cells. Immunoprecipitation assays were performed first with nuclear extracts from C8161 cells and HEM human primary melanocytes using antibodies against Sp1, Sp2 and Sp3. The immuno-complexes were subjected to Western blot with NRSF antibody. Both C8161 and HEM showed NRSF expression; only C8161 showed binding of NRSF to Sp1. **C:** Western blot of C8161, UACC903, M19 and MV3 cells treated with 100nM and 500nM mithramycin A for 24 hours and probed for mGluR1 expression normalized to α -Tubulin. The numbers represent arbitrary units of intensity of mGluR1 to α -Tubulin determined with Optiquant Software. **D:** Western blots of C8161 and M19 cells transiently transfected with siSP1 after 72 hours, protein lysates were prepared and probed for Sp1 expression (left panel). Same lysates probed for mGluR1 expression and normalized to α -Tubulin (right panel). The numbers represent arbitrary units of intensity of mGluR1 to α -Tubulin determined with Optiquant Software.

Figure 4.

A: Western immunoblots of cell lysates isolated from hTERT/CDK4^{R24C}/p53^{DD} and **B:** FOM 139-1 cells on day 0, 3, 5, 7, or 9 after 5-azacytidine treatments and probed with antibody to mGluR1 with mGluR1-positive melanoma SK-Mel187 cells as positive control. α-Tubulin was used to show equal loading. MAGE A4 expression was used as a positive control for demethylated DNA. C8161 human melanoma cell lysates were used as positive control for MAGEA4. α-Tubulin was used to show equal loading. **C:** Promoter methylation PCR assays were performed with genomic DNA isolated from hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 according to manufacturer's instruction. Left panel; PCR performed with primer set flanking *GRM1* promoter region (bases -4846/-5267) and eluted complexes of MeCP2 and Mse1 digested hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 genomic DNA. M, marker, Hae III digested ϕ x174 DNA, Lanes 1–3 are hTERT/CDK4^{R24C}/p53^{DD}; no DNA control, Mse1 digested genomic DNA, MeCP2 complexed Mse1 digested DNA. Lanes 4–6 are FOM 139-1; no DNA control, Mse1 digested genomic DNA, MeCP2 complexed Mse1 digested DNA. Right panel, PCR performed with primer set flanking *GRM1* promoter region (bases -3257/-3874) and eluted complexes of MeCP2 and Mse1 digested hTERT/CDK4^{R24C}/p53^{DD} and FOM 139-1 genomic DNA, lanes 7–9 are hTERT/CDK4^{R24C}/p53^{DD}; no DNA control, Mse1 digested genomic DNA, MeCP2 complexed Mse1 digested DNA. Lanes 10–12 are FOM 139-1; no DNA control, Mse1 digested genomic DNA, MeCP2 complexed Mse1 digested DNA.

Table 1

NRSE and Sp1 binding sites collectively repress *GRM1* promoter activity in C8161 and UACC903 human melanoma cells and normal human primary melanocytes (HEM) but much less in HEK293 cells. All cells were transfected with wild type NRSE/SP1 (hGRM1NESP), mutated NRSE wild type SP1 (hGRM1NEmSP), wild type NRSE mutated Sp1 (hGRM1NESPm), or mutated at both NRSE and SP1 (hGRM1NEmSPm) linked luciferase reporter gene constructs together with Renilla luciferase. The promoter activity of each construct is expressed as described in Figure 2. All luciferase assays were repeated three times and the results shown are the average of all three experiments. Significance was determined by using unpaired Student's 2-tailed *t* test. A *p* value of 0.05 was considered significant.

Recombinant constructs of NRSE and Sp1	HEK293	HEM	C8161	UACC903
hGRM1NESP (Relative Luciferase Activity, fold)	33.07±1.08	19.1±1.57	53.1±1.62	93.3±5.75
hGRM1NEmSP (Relative Luciferase Activity, fold)	79.81±2.42 *	39±2.85 *	63.98±9.09	138.42±8.63 *
hGRM1NESPm (Relative Luciferase Activity, fold)	29.2±1.96	17.5±0.67	18.15±0.32 *	25.56±4.48 *
hGRM1NEmSPm (Relative Luciferase Activity, fold)	66.12±0.04 *	12.3±2.51 **	40.00±0.74 **	79.61±3.27 **

*, *p*<0.01,

**, *p*<0.04, compared to wild type hGRM1NRSE/Sp control.